AEM
Journals.ASM.org

Real-Time PCR and Sequencing Assays for Rapid Detection and Identification of Avian Schistosomes in Environmental Samples

Narayanan Jothikumar,[a] Bonnie J. Mull,[b] Sara V. Brant,[c] Eric S. Loker,[c] Jeremy Collinson,[d] W. Evan Secor,[e] Vincent R. Hill[a]

Waterborne Disease Prevention Branch, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[a]; Food and Waterborne Disease Program, Bureau of Epidemiology, Florida Department of Health, Tallahassee, Florida, USA[b]; Department of Biology, COBRE Center for Evolutionary and Theoretical Immunology, Museum of Southwestern Biology, Division of Parasitology, University of New Mexico, Albuquerque, New Mexico, USA[c]; Central District Health Department, Grand Island, Nebraska, USA[d]; Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, Georgia, USA[e]

Cercarial dermatitis, also known as swimmer's itch, is an allergenic skin reaction followed by intense itching caused by schistosome cercariae penetrating human skin. Cercarial dermatitis outbreaks occur globally and are frequently associated with freshwater lakes and are occasionally associated with marine or estuarine waters where birds reside year-round or where migratory birds reside. In this study, a broadly reactive TaqMan assay targeting 18S rRNA gene (ribosomal DNA [rDNA]) sequences that was based on a genetically diverse panel of schistosome isolates representing 13 genera and 20 species (the 18S rDNA TaqMan assay) was developed. A PCR assay was also developed to amplify a 28S rDNA region for subsequent sequencing to identify schistosomes. When applied to surface water samples seeded with *Schistosoma mansoni* cercariae, the 18S rDNA TaqMan assay enabled detection at a level of 5 *S. mansoni* cercariae in 100 liters of lake water. The 18S rDNA TaqMan and 28S rDNA PCR sequencing assays were also applied to 100-liter water samples collected from lakes in Nebraska and Wisconsin where there were reported dermatitis outbreaks. Avian schistosome DNA was detected in 11 of 34 lake water samples using the TaqMan assay. Further 28S rDNA sequence analysis of positive samples confirmed the presence of avian schistosome DNA and provided a preliminary identification of the avian schistosomes in 10 of the 11 samples. These data indicate that the broadly schistosome-reactive TaqMan assay can be effective for rapid screening of large-volume water samples for detection of avian schistosomes, thereby facilitating timely response actions to mitigate or prevent dermatitis outbreaks. Additionally, samples positive by the 18S rDNA TaqMan assay can be further assayed using the 28S rDNA sequencing assay to both confirm the presence of schistosomes and contribute to their identification.

In recent years, waterborne outbreaks of cercarial dermatitis, also known as swimmer's itch, have increasingly been reported worldwide (1–11). Cercarial dermatitis is caused by free-swimming cercariae of avian and mammalian schistosomes emerging from snails that penetrate and die within human skin, causing an inflammatory immune response (12). The symptoms are clinically recognized by initial erythema and cutaneous itch and then subsequent macular and papular eruptions (12, 13). Although several factors can favor the presence of thriving snail populations and increase the risk of dermatitis outbreaks, one specific circumstance that contributes is the prolonged presence of high-density bird populations in lakes, resulting in eutrophication due to the feces deposited in the water (8, 14). Eutrophication encourages the growth of aquatic vegetation, providing an abundance of food and thus increasing the snail and wild bird populations needed to support the schistosome life cycle (10, 15).

There are more than 100 species of schistosomes in 14 recognized genera, with new genera and species still being reported (16, 17). Schistosomes have a two-host life cycle, with a mammal or bird definitive host and an aquatic snail intermediate host. Cercarial dermatitis is caused by both mammalian and avian schistosomes, though most cases reported involve avian schistosomes (18). Most adult avian schistosomes live in the mesenteric and portal vessels of aquatic birds and produce eggs that are excreted in bird feces. One exception is *Trichobilharzia regenti*, which lives and produces eggs in the nasal passages of birds in Europe (19). Once in the water, the eggs hatch and release miracidia that infect the snail host. Miracidia transform into primary sporocysts within the snails, and these produce daughter sporocysts in which cercariae are produced. Cercariae are released or shed from snails and are the free-swimming infective stage. Normally, these cercariae would penetrate the skin of wild birds, often waterfowl. However, when human skin is exposed to cercaria-contaminated water, the cercariae penetrate. This happens because avian schistosomes are attracted to general fatty acids shared by both avian and mammalian skin (20–22). Because humans are an inappropriate host of avian schistosomes, the parasites die in the skin and host immune responses to them cause dermatitis. The schistosome genus *Trichobilharzia* is most often implicated in swimmer's itch outbreaks not just in North America but also worldwide (8, 23). About 40 species of *Trichobilharzia* have been recognized and are reported mostly from ducks (3). Here it must be noted that species of *Trichobilharzia* are often assumed to be the cause of outbreaks, even though no specific means of identification have been provided. It is prudent to consider that any schistosome may be ca-

Received 5 March 2015 **Accepted** 7 April 2015
Accepted manuscript posted online 10 April 2015
Citation Jothikumar N, Mull BJ, Brant SV, Loker ES, Collinson J, Secor WE, Hill VR. 2015. Real-time PCR and sequencing assays for rapid detection and identification of avian schistosomes in environmental samples. Appl Environ Microbiol 81:4207–4215. doi:10.1128/AEM.00750-15.
Editor: M. W. Griffiths
Address correspondence to Narayanan Jothikumar, jin2@cdc.gov.

doi:10.1128/AEM.00750-15

pable of causing dermatitis, and as several schistosome genera are widely distributed around the world (23), further discrimination of the genera and species involved in causing specific outbreaks is needed.

Outbreaks of cercarial dermatitis can have significant economic repercussions on the local water recreation industry within affected communities, as beaches and recreational areas are often closed to protect public health. There is a need for research efforts to develop water testing and molecular tools to enable state public health and natural resources agencies to investigate suspected outbreaks. This in turn will lead to a better understanding of avian schistosome ecology in lakes that are repeated sources of swimmer's itch outbreaks and to the development of interventions to prevent them. The current practice of identification of avian schistosomes relies on conventional PCR (3, 24, 25) and has certain limitations for routine diagnosis due to the necessary product confirmation step using agarose gel electrophoresis, which may result in carryover contamination. In 2002, Hertel et al. (26) developed a conventional PCR method for the detection of avian schistosomes, and the reported assay was evaluated with surface water samples by Schets et al. (27). Real-time PCR assays offer several advantages for the rapid detection of parasites over conventional PCR, including closed-tube amplification, high throughput, rapid amplification times, and elimination of the need for post-PCR manipulation. Currently, no real-time PCR assays for the detection of avian schistosomes have been published, and there is a pressing need for the development of a highly sensitive real-time PCR-based assay for the detection of the low numbers of cercariae often present in water samples.

The present study focuses on the development of an 18S rRNA gene (ribosomal DNA [rDNA])-specific PCR-based assay, to detect avian schistosomes in water samples, coupled with a follow-up 28S rDNA-specific PCR and sequencing, to identify the schistosomes to the species or genus level. This study capitalizes on vouchered museum specimens collected from five different continents to provide a comparison of the most diverse panel of avian schistosome genera accumulated to date. The new molecular testing methodology was used in studies of two lakes, one in Nebraska and one in Wisconsin, with a history of reported swimmer's itch cases.

MATERIALS AND METHODS

Schistosome specimens for assay development. The real-time PCR assay was developed using a panel of 21 isolates of schistosomes. Schistosomes and two other nonschistosome species used for the study were derived from the collections of and were characterized by the Division of Parasites, Museum of Southwestern Biology, University of New Mexico. The avian schistosomes were avian schistosome B, avian schistosome C, avian schistosome D, *Allobilharzia visceralis*, *Anserobilharzia brantae*, *Austrobilharzia variglandis*, *Ornithobilharzia canaliculata*, California cercariae, *Dendritobilharzia pulverulenta*, *Gigantobilharzia huronensis*, *Trichobilharzia physellae*, *Trichobilharzia querquedulae*, *Trichobilharzia stagnicolae*, *Trichobilharzia* sp. strain A, *Trichobilharzia* sp. strain E, *Trichobilharzia* sp. strain C, and *Trichobilharzia* nasal species similar to *T. regenti*. The mammalian schistosomes were *Schistosomatium douthitti*, *Schistosoma nasale*, and *Schistosoma spindale*. This collection represents a total of 13 genera and 20 species of schistosomes. Additionally, DNA from cercariae from two species of common, nonschistosome trematodes was also used in this study: an *Echinoparyphium* sp. (Echinostomatidae) and a strigeid trematode (Strigeidae). Cercariae of the human schistosome *Schistosoma mansoni* obtained from CDC were also included in the study. For testing of the specificity of the TaqMan assay, DNA samples extracted from three species of laboratory-raised noninfected snails were also examined: *Helisoma trivolvis* (Planorbidae), *Physa acuta* (Physidae), and *Radix auricularia* (Lymnaeidae). DNA extracts from the parasitic nematode *Angiostrongylus cantonensis* and some of its snail/slug hosts, including *Achatina fulica*, a *Pomacea* sp., *Zachrysia provisoria*, *Alcadia striata*, *Bulimulus guadalupensis*, *Bradybaena similaris*, *Veronicella cubensis*, a *Pallifera* sp., *Euglandina rosea*, and *Parmarion martensi*, were also tested. The specificity panel also included DNA extracts from the parasites *Necator americanus*, *Strongyloides stercoralis*, a *Taenia* sp., *Trichuris trichiura*, *Ascaris lumbricoides*, *Ascaris suum*, *Balamuthia mandrillaris*, *Naegleria fowleri*, *Acanthamoeba keratitis*, *Toxoplasma gondii*, *Entamoeba histolytica*, *Entamoeba dispar*, *Cryptosporidium parvum*, and *Giardia duodenalis*. For schistosome, strigeid, and echinostome specimens, DNA was extracted from nine ethanol-preserved cercariae or a portion of adult worms using HotShot lysis (28).

Sequence analysis of 18S rDNA fragment. Prior to development of the 18S rDNA-specific TaqMan assay (the 18S rDNA TaqMan assay), all isolates were sequenced to identify conserved regions among avian schistosomes for the design of primers and probes. A pair of primers (primer 18S-JVSQF [ACGGGTAACGGGGAATCA, positions 371 to 388] and primer 18S-JVSQR [ACTTCGGATCCGAAAACCAACA, positions 970 to 949]) was designed to obtain sequence data from schistosome DNA to identify the conserved region for the TaqMan assay. The real-time PCR was performed using an intercalating dye (SYTO 9) to monitor fluorescence during amplification. The real-time PCR assay mixture consisted of a 20-μl final reaction volume containing TaqMan environmental master mix (version 2.0) PCR buffer (Life Technologies, Grand Island, NY) with 0.25 μM each forward primer (18S-JVSQF) and reverse primer (18S-JVSQR), 1.25 μM SYTO 9, and 2 μl DNA sample. The amplification reaction conditions were 10 min at 95°C, followed by 50 cycles of denaturation for 15 s at 95°C and annealing for 40 s at 60°C and then fluorescence acquisition at the end of annealing for the monitoring of the fluorescent signal during the amplification. The amplified products were purified from 23 isolates and sequenced using Sanger DNA sequencing (GeneWiz, South Plainfield, NJ). The amplified PCR product was sequenced with forward sequencing primer 18S-JVSQP (TTCGATTCCGG AGAGGGAGCCT, specific for residues 392 to 413, or approximately 570 bp). The positions indicated above are based on the *Allobilharzia visceralis* sequence with GenBank accession number EF114220.

Design of TaqMan assay. On the basis of the 18S rDNA sequence information and alignment for 23 DNA samples obtained using Clone Manager software (version 9), a TaqMan assay was manually designed to target a highly conserved region. The primers and probe were designed in the homologous region to maximize the detection of cercariae (Fig. 1), resulting in amplification of a 154-bp fragment of the 18S rDNA region. The sequences of the forward primer (primer JVSF), reverse primer (primer JVSR), and TaqMan probe (JVSP) were AGCCTTTCAGCCGTA TCTGT (positions 742 to 761), AGGCCTGCCTTGAGCACT (positions 895 to 878), and 5′-TCGGGAGCGGACGGCATCTTTA-3′ (positions 841 to 862), respectively. The positions in parentheses are based on the *Allobilharzia visceralis* sequence with GenBank accession number EF114220. The TaqMan probe was labeled with 6-carboxyfluorescein (FAM) at the 5′ end and with black hole quencher 1 (BHQ-1) at the 3′ end. The designed primers and probe were evaluated for specificity through BLAST searches of the sequences in the GenBank database for comparison with target sequences.

Evaluation of TaqMan assay. The TaqMan assay was evaluated for specificity using a set of genomic DNA extracts from 21 schistosome and 2 nonschistosome isolates. The TaqMan assay mixture consisted of a 20-μl final reaction volume containing TaqMan environmental master mix (version 2.0) PCR buffer with 0.25 μM each forward and reverse primers, 0.1 μM TaqMan probe, and 2 μl DNA sample. The amplification reactions for the TaqMan assay were performed on an ABI 7500 (Applied Biosystems, CA) platform. Cycling conditions were as follows: 10 min at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C and annealing

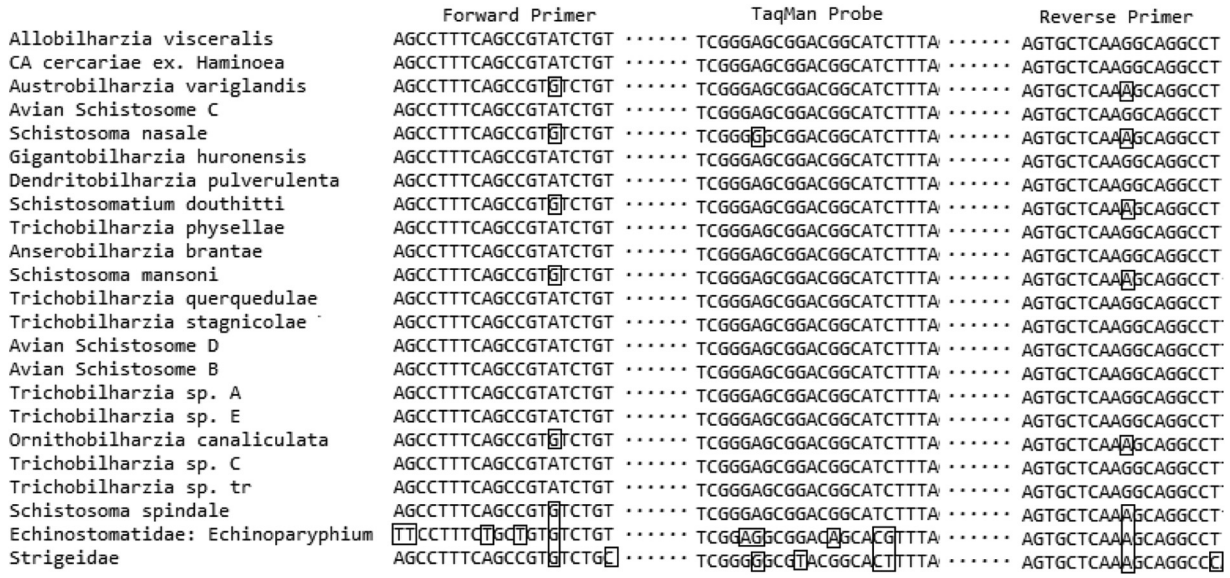

FIG 1 Alignment of highly conserved regions of schistosome 18S rDNA sequences for determination of the sequences of the primers and TaqMan probe used in the study. The actual sequence of the reverse primer is 5′-AGGCCTGCCTTGAGCACT-3′. Mismatched nucleotides are shown in boxes. The dots between sequences represent gaps in the sequence alignment.

for 40 s at 60°C and then fluorescence acquisition at the end of annealing for the monitoring of the fluorescent signal during the amplification. For reproducibility, triplicate samples were tested at each dilution. Negative controls were included for every reaction setup. The detection limit was established on the basis of the highest dilution with a positive signal. A sample was considered positive on the basis of the cycle threshold (C_T) values. The sample was considered negative if the C_T value was 40 or higher.

The sensitivity of the TaqMan assay was evaluated using a standard curve prepared using two sets of stocks containing 1, 2, 5, and 10 cercariae. Known numbers of *S. mansoni* cercariae were counted using microscopy and harvested into 1.5-ml microcentrifuge tubes containing 500 μl of 100% ethanol. Into each tube containing 1, 2, 5, or 10 *S. mansoni* cercariae was mixed an equal amount of 2× lysis buffer (universal nucleic acid extraction [UNEX] buffer; Microbiologics, St. Cloud, MN) containing proteinase K for DNA extraction, followed by bead beating and DNA purification using a silica spin column, as previously reported (29). Schistosome DNA was eluted with 100 μl of TE (Tris-EDTA), and 2 μl was assayed by real-time PCR (18S rDNA TaqMan assay). Thus, the cercaria equivalent per PCR mixture was 1/50 of the number of cercaria in each stock used for the standard curve analyses. All DNA specimens were assayed in duplicate.

28S rDNA sequencing assay for avian schistosome identification. A pair of primers (primer 28S-JVSQF [GTCTGCTTGTCRGTGCACTTTC, positions 565 to 586] and primer 28S-JVSQR [ACATGTTAAACTCCTT GGTCCGTGT, positions 954 to 930]) was designed to obtain sequence data from the 21 schistosome and 2 nonschistosome isolates, snail DNA, and water samples to confirm that the TaqMan assay could detect the various schistosome species. This primer pair targets a region of the 28S rDNA sequence (~390 bp) for identification of the cercariae to the species level. The positions presented in parentheses above are based on the sequence of *Gigantobilharzia melanoidis* (GenBank accession number JX875068). Real-time PCR was performed using an intercalating dye (SYTO 9). The real-time PCR assay mixture consisted of a 20-μl final reaction volume containing TaqMan environmental master mix (version 2.0) PCR buffer with 0.25 μM each forward primer (28S-JVSQF) and reverse primer (28S-JVSQR), 1.25 μM SYTO 9, and 2 μl DNA sample. The amplification reaction conditions were as follows: 10 min at 95°C, followed by 50 cycles of denaturation for 20 s at 95°C and annealing for 40 s at 60°C and then fluorescence acquisition at the end of annealing. The products amplified from 23 isolates were purified and sequenced using Sanger DNA sequencing (GeneWiz, South Plainfield, NJ). The amplified product was sequenced using primer 28S-JVSQP (TCACCACGACCGG, positions 596 to 608). Sequencing results that contained any ambiguous bases or double peaks were discarded. The sequences of the PCR products were subjected to a BLAST search and were assigned to a genus or species only when similarity scores of 95% or above were obtained.

To provide further orientation regarding the identity of unknown schistosomes from environmental samples, sequences were placed in a phylogenetic tree constructed using a maximum likelihood algorithm in PAUP* (version 4.0b10) (30) and the sequences of avian schistosome species available in GenBank.

Detection of cercariae in water samples using DEUF and TaqMan assay. One hundred-liter water samples (turbidity, 90 nephelometric turbidity units) were collected from Murphey Candler Park (MCP) Lake in Atlanta, GA. *Schistosoma mansoni* cercariae were counted by microscopy and seeded into 100-liter water samples at levels of 1,000, 12, 5, and 1 cercariae. Replicate experiments were performed at each seed level (except at the level of 1,000 cercariae), as well as for background (nonseeded) samples. Water samples were concentrated by dead-end ultrafiltration (DEUF) as previously described (31). The 500-ml back-flush solution was further concentrated by centrifugation for 30 min at 4,000 × *g*. After removing the supernatant, the volume of the resuspended pellets was ~4 ml. A portion of 750 μl from each pellet was subjected to the nucleic acid extraction procedure described by Hill et al. (29). Briefly, this method was comprised of (i) lysis using a chaotropic buffer, proteinase K, and bead beating and (ii) separation and purification of DNA using a silica spin column and a polyvinylpolypyrrolidone (PVPP) spin column (Zymo-Spin IV-HRC column; Zymo Research Corporation, Orange, CA), resulting in a final extracted nucleic acid volume of 100 μl in TE buffer. The PCR mixtures included 5 μl of the extracted DNA, 2× TaqMan environmental master mix (version 2.0; Life Technologies, Grand Island, NY), primers (0.25 μM each), a probe (0.1 μM) for 18S rDNA or 1.25 μM SYTO 9 dye for 28S rDNA, nonacetylated bovine serum albumin (400 ng/μl), T4 phage gene 32 protein (25 ng/μl), and water, which was added so that the final volume was 50 μl. Each PCR master mix also included a TaqMan exogenous internal positive control (IPC; Life Technologies, Grand Island, NY) for quality control (identification of false-negative results and evaluation of potential PCR inhibition). PCR was performed

FIG 2 Photo of Mormon Island Lake, Nebraska, on 14 March 2013 showing tens of thousands of migrating snow geese. The sand beach recreational area is shown on the far side of the lake.

under the following thermal cycling conditions for both targets: an initial denaturation step at 95°C for 10 min and 45 cycles of amplification (95°C for 10 s, 60°C for 40 s). Duplicate PCR assays were performed for each water sample.

Application of molecular assays to lake water samples. In 2012, suspected cases of swimmer's itch associated with lakes in Nebraska and Wisconsin were reported to CDC. Water samples were collected from the beaches of known swimming areas at both lakes to determine if schistosome cercariae could be detected in the water of the lakes associated with the reported cases. Three water samples were collected from Mormon Island Lake, Nebraska, in June 2012, and four water samples were collected from Twin Lake, Wisconsin, in August 2012. At each sampling location, a 100-liter water sample was filtered in the field through a Rexeed-25 SX dialyzer (Asahi Kasei Medical, Japan) using dead-end ultrafiltration (31). Ultrafilters (i.e., dialyzers) were chilled, shipped to the analytical laboratory at CDC (Atlanta, GA), and processed within 24 h of sampling. The ultrafilters were flushed according to the method of Smith and Hill (31), resulting in concentrated samples with an average volume of approximately 450 ml. The samples were further concentrated by centrifugation at 4,000 × *g* for 30 min. The DNA in the pelleted sample was extracted using a commercially available 2× lysis buffer (UNEX buffer; Microbiologics, St. Cloud, MN) and a bead beating procedure as previously reported (29), and 5 μl of the DNA extract was assayed by the TaqMan assay targeting the 18S rDNA and the 28S rDNA assay for sequencing, in duplicate. Any environmental samples that were positive by the 18S rDNA TaqMan assay (samples with C_T values of 40 or less) were subjected to 28S rDNA amplification for sequencing.

In March 2013, the 42-acre (170,000-m^2) Mormon Island Lake was visited over a 3-week period by tens of thousands of migrating geese (Fig. 2). Over the course of 3 months (from approximately April to June), CDC and the Nebraska Central District Health Department collaborated to systematically monitor when cercariae could be detected and identify the schistosome species in the lake samples. Snails and 50-liter ultrafiltration water samples were collected from 28 March to 24 June 2013. Ultrafiltered samples were collected every 2 weeks from three sites along the swimming beach. At the time of sample collection, observations of the quantity of geese, snails, and fish beds were also recorded. A snail specimen collected from Mormon Island Lake was identified as *Physa acuta* on the basis of the morphology. Water samples were analyzed by PCR (the 18S rDNA TaqMan assay and 28S rDNA assay for sequencing) in duplicate, and 28S rDNA from positive samples was sequenced to identify the avian schistosome species. In 2014, Mormon Island Lake did not experience the usual large goose migration seen in previous years. Nevertheless, incidents of suspected swimmers itch were reported beginning in May. Water samples for filtration, concentration, and PCR were collected in May and June to verify whether cercariae could be detected.

Nucleotide sequence accession numbers. The schistosome and nonschistosome 28S rDNA sequences obtained in this study (see Table 1) have been deposited in the GenBank database under accession numbers KP734292 to KP734314, corresponding to the list of isolates in Table 1.

RESULTS

Design of TaqMan assay on the basis of 18S rDNA sequence analysis. The array of schistosome species used in this study covers much of the breadth of diversity in the Schistosomatidae (23) available, including seven known and four not yet fully characterized avian schistosome genera and seven distinct *Trichobilharzia* lineages. The schistosomes in these samples represented both the major basal and derived avian schistosome clades and included species from both marine and freshwater habitats with representation from multiple continents. Mammalian schistosomes from both North America and Asia implicated in causing dermatitis were also included. All of the 23 cercarial isolates were initially sequenced for verification based on real-time PCR amplification with SYTO 9 and a pair of primers (18S-JVSQF/18S-JVSQR) targeting the 18S rDNA region. On the basis of sequence analysis, primers and probes (TaqMan) that anneal to highly conserved regions of 18S rDNA were designed (Fig. 1).

Specificity of 18S rDNA TaqMan assay. The 18S rDNA TaqMan assay generated C_T values for each isolate ranging from 19 to 33 (Table 1). No cross-reactivity with DNA extracted from *Echinoparyphium*, strigeid cercariae, noninfected freshwater snails

TABLE 1 Real-time PCR results and C_T values for evaluating the specificity of the 18S and 28S rDNA PCR assays

Specimen identification no.	Isolate	C_T value	
		18S rDNA TaqMan assay	28S rDNA SYTO 9 sequencing assay
MSB:Para:18550	*Allobilharzia visceralis* W246	19.5	20.9
MSB:Para:18669	California cercariae ex. *Haminoea* sp. strain W324	21.3	25.3
MSB:Para:18534	*Austrobilharzia variglandis* W357	32.8	25.7
MSB:Para:18681	Avian schistosome C W403	25.6	32.0
MSB:Para:19069	*Schistosoma nasale* W439	32.9	29.3
MSB:Para:18697	*Gigantobilharzia huronensis* W446	19.3	21.5
MSB:Para:18702	*Dendritobilharzia pulverulenta* W499	24.5	26.3
MSB:Para:19073	*Schistosomatium douthitti* W514	32.8	26.5
MSB:Para:18657	*Trichobilharzia physellae* W235	20.9	22.2
MSB:Para:14743	*Anserobilharzia brantae* W331	26.3	29.6
CDC	*Schistosoma mansoni*	25.0	19.9
MSB:Para:18588	*Trichobilharzia querquedulae* W154	21.0	21.4
MSB:Para:18650	*Trichobilharzia stagnicolae* W222	23.6	24.9
MSB:Para:18683	Avian schistosome D W405	25.3	26.3
MSB:Para:18688	Avian schistosome B W409	30.2	31.4
MSB:Para:18706	*Trichobilharzia* sp. A W511	19.2	19.8
MSB:Para:18618	*Trichobilharzia* sp. E W337	21.8	23.9
MSB:Para:18542	*Ornithobilharzia canaliculata* W393	27.6	21.7
MSB:Para:18563	*Trichobilharzia* sp. C W174	23.2	25.7
MSB:Para:19072	*Trichobilharzia* sp. strain tr W7077	24.7	24.9
MSB:Para:19071	*Schistosoma spindale* W558	31.7	23.4
MSB:Para:19074	Echinostomatidae: *Echinoparyphium* strain PS12	Neg.[a]	26.5
MSB:Para:19075	Strigeidae strain PS19	Neg.	30.4

[a] Neg., negative by the 18S rDNA TaqMan assay.

(*Helisoma trivolvis*, *Physa acuta*, and *Radix auricularia*), the parasitic nematode *Angiostrongylus cantonensis* (or any of the terrestrial gastropod hosts of this nematode), or any other nonschistosome helminths or protozoans tested was observed (not shown in Table 1). Both the primers and the probe selected were more specific to schistosomes, based on confirmation using BLAST searches of the sequences in the GenBank database. The TaqMan assay resulted in the highly specific detection of schistosomes without any false-positive results. All control DNA remained negative in each run.

Sensitivity of TaqMan assay. The limit of detection of *S. mansoni* by the TaqMan assay was determined. The standard curves for the assay were linear when the C_T values were plotted against the starting cercarial equivalent per reaction (Fig. 3). An R^2 value of 0.96 and a slope of −3.1 were obtained for the TaqMan assay. This corresponds to calculated PCR efficiencies of 110%. A cercaria equivalent of 0.02 cercaria per reaction was determined to be the detection limit for the assay.

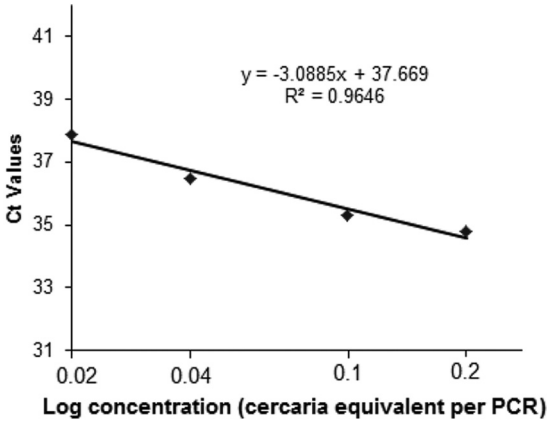

FIG 3 Standard curve for TaqMan assay using *S. mansoni* cercaria DNA.

28S rDNA sequencing for identification. The C_T values for the 28S rDNA real-time PCR assay ranged from 19 to 32 for the cercarial isolates that were positive using the 18S rDNA TaqMan assay (Table 1). All 28S rDNA PCR amplification products from the 21 schistosome and 2 nonschistosome species were sequenced, and the sequences were matched to the known available sequences (in GenBank) for each isolate.

Detection limit of experimental method using seeded lake water. Replicate experiments performed at multiple cercaria seeding levels indicated that the use of the 18S rDNA TaqMan PCR assay in conjunction with DEUF enabled avian schistosome detection at a level of 5 *Schistosoma mansoni* cercariae in 100 liters of surface water (Table 2). For experiments on nonseeded lake water samples, no cercariae were detected. No appreciable PCR inhibition was observed, based on comparison of the C_T values for the internal control (IC) in assays of DNA from water concentrates (C_T range = 26.6 to 26.8) and those in the assay with the no-template controls (average C_T value = 26.8).

Application of TaqMan assay and sequencing analysis to lake water samples. All three swimmers itch-related water samples collected at Mormon Island Lake (Nebraska) in 2012 were negative by the 18S rDNA TaqMan assay (Table 3). In contrast, two of the four water samples collected from Twin Lake (Wisconsin) in 2012 were positive. The assay results for each duplicate sample were the same (i.e., both were negative or both were positive).

TABLE 2 Recovery and detection of seeded *S. mansoni* cercariae in surface water by the combination of DEUF and 18S rDNA TaqMan PCR

Cercaria seed level	Positive detection rate by 18S rDNA TaqMan assay[a]	Avg C_T value by 18S rDNA TaqMan assay	IC C_T value
1,000	1/1	30.0	26.7
12	2/2	34.9	26.6
5	2/2	39.4	26.6
1	0/2	Neg.[b]	26.8
0	0/2	Neg.	26.7

[a] Data represent the numbers of samples positive/total number of samples tested.
[b] Neg., negative by the 18S rDNA TaqMan assay.

Double-stranded sequencing sometimes resulted in mixed results, possibly as the result of the presence of a mixture of species in the environmental samples, so only a single-stranded sequencing approach was selected for such samples. Using the 28S rDNA sequencing assay, a schistosome with a sequence closely related to that of *Trichobilharzia physellae* was identified (Fig. 4) in one Twin Lake sample (Wisc6024) and an opisthorchiid with a sequence similar to that of *Clonorchis sinensis* was identified in another sample (Wisc6026).

During the 3-month study of Mormon Island Lake in 2013, 7 of 24 samples were positive by the 18S rDNA TaqMan assay. The 28S rDNA sequencing assay yielded sequences that allowed further characterization for all seven 18S rDNA TaqMan assay-positive samples (Table 4). Six of the sequences clustered with *Gigantobilharzia huronensis* in a BLAST analysis of the sequences in GenBank and grouped with avian schistosome B (unpublished data, sequence not in GenBank) in the phylogenetic analysis (Neb0529Middle, Neb0611Middle, Neb0619Middle, Neb0619West, Neb0624West, and Neb0624Middle; Fig. 4). The sequence of the seventh 18S rDNA TaqMan assay-positive sample (Neb0624South) was similar to the sequences of several species of *Trichobilharzia* in a BLAST analysis of the sequences in GenBank but did not group with any known species of *Trichobilharzia* in the phylogenetic analysis. In 2014, two of the three samples collected from Mormon Island Lake following reports of suspected swimmer's itch were positive by the 18S rDNA TaqMan assay. The 28S rDNA sequencing assay indicated that these samples contained *Trichobilharzia* spp. (Table 5) that are associated with a group of taxa including *Trichobilharzia querquedulae* (3).

TABLE 3 Avian schistosome detection by 18S rDNA TaqMan assay in lake water samples collected in Nebraska and Wisconsin[a]

Sample identification no.	18S rDNA TaqMan assay result	Identification based on 28S rDNA sequencing
Neb6015	Neg.	None
Neb6016	Neg.	None
Neb6017	Neg.	None
Wisc6024	Pos.	*Trichobilharzia* sp.
Wisc6025	Neg.	None
Wisc6026	Pos.	Opisthorchiid-like cercaria
Wisc6027	Neg.	None

[a] Samples were collected in 2012. Neg., negative by the 18S rDNA TaqMan assay; Pos., positive by the 18S rDNA TaqMan assay; None, no sequencing information was obtained. Only samples that were positive by the TaqMan assay were subjected to the 28S rDNA real-time PCR assay with SYTO 9 and sequenced. See Fig. 4 for phylogenetic identification.

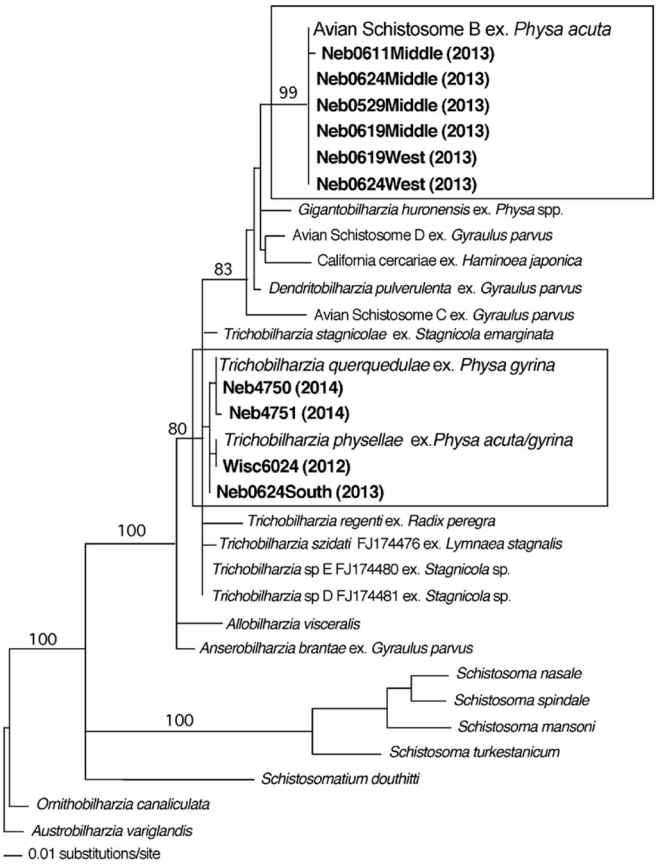

FIG 4 Phylogenetic tree based on 28S rDNA sequences. Samples in bold are from the environmental samples collected for this study, and the year in which the sample was collected follows the sample identifier. For the avian schistosomes, the GenBank accession number and/or the snail (ex.) from which it was collected, where known, follows the taxonomic name. If there is no GenBank number, then the samples were from this study and are described in Table 1. The boxes indicate where the water samples grouped with previously identified avian schistosomes, *Trichobilharzia*, and a yet to be characterized lineage of avian schistosome B.

DISCUSSION

Avian schistosomes are important pathogens of birds and can cause cercarial dermatitis in humans exposed during recreational bathing. Given the abundance of species of avian schistosomes and their widespread occurrence, including those that cause cercarial dermatitis, it is important to develop molecular assays to monitor their presence in environmental water samples. Such tools will enable public health scientists to understand their temporal distribution, evaluate exposure risks for bathers, and understand the associated pathogenicity related to skin infections.

The development of accurate means of identification of avian schistosomes using molecular tools is quite challenging, as more than 100 species have been identified, and more are going to be found. The traditional morphology-based identification of cercariae is impractical in most cases. With respect to molecular identification, although considerable progress in revealing a much broader representation of the schistosome diversity present has been made, more progress is needed to provide definitive se-

TABLE 4 Avian schistosome detection in water samples collected at Mormon Island Lake, Nebraska[a]

Sampling site	Result							
	28 March	15 April	29 April	13 May	29 May	11 June	19 June	24 June
South	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos. (Tr)
Middle	Neg.	Neg.	Neg.	Neg.	Pos. (GHL)	Pos. (GHL)	Pos. (GHL)	Pos. (GHL)
West	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	Pos. (GHL)	Pos. (GHL)

[a] Samples were collected in 2013. Neg., negative by the 18S rDNA TaqMan assay; Pos., positive by the 18S rDNA TaqMan assay. *Trichobilharzia* spp. (Tr) and avian schistosome clade B, which is a *Gigantobilharzia huronensis* (GHL)-like organism, were identified by 28S rDNA sequencing and comparison with the sequences in the GenBank database by BLAST analysis to be the most similar match for environmental samples Neb0529Middle (which tested positive for *Gigantobilharzia huronensis*), Neb0611Middle (which tested positive for *Gigantobilharzia huronensis*), Neb0619Middle (which tested positive for *Gigantobilharzia huronensis*), Neb0619West (which tested positive for *Gigantobilharzia huronensis*), Neb0624South (which tested positive for *Trichobilharzia*), Neb0624Middle (which tested positive for *Gigantobilharzia huronensis*), and Neb0624West (which tested positive for *Gigantobilharzia huronensis*). See Fig. 4 for phylogenetic identification.

quence-based identifications, particularly for the fast-evolving genus *Trichobilharzia* (23). Nonetheless, it is now possible to use sequence-based approaches to identify the genera, species groups, or, in some cases, particular species that are implicated in causing dermatitis outbreaks. Sequence-based approaches can now be coupled with advances made in methods for collecting large-volume environmental water samples to offer a feasible way for screening habitats at different points and times for the potential for outbreaks.

Consequently, for this study, 21 isolates representing named species or genetically distinct lineages across the phylogenetic tree for the Schistosomatidae were selected to provide a representative overview of potential causative agents of dermatitis and were then characterized for both selected 18S and 28S rDNA sequences. A broadly schistosome-reactive universal TaqMan assay was designed as a sensitive and specific screening assay to rapidly detect the presence of cercariae in water. This 18S rDNA TaqMan assay was found to be slightly more specific than the 28S rDNA PCR assay (Table 1) and was determined to be highly sensitive (it was able to detect the equivalent of 0.02 cercaria equivalents per PCR [Fig. 3]). When applied to surface water samples, the 18S rDNA TaqMan assay yielded positive detections for 100-liter water samples seeded with 5 *S. mansoni* cercariae. The 18S rDNA TaqMan assay developed for the direct detection of cercariae in environmental samples did not cross-react with DNA extracted from snails or nonseeded lake water samples.

A total of 34 samples were collected from two lakes where suspected cases of swimmer's itch had been reported. Using the 18S rDNA TaqMan PCR screening assay, 11 of 34 samples (32%) were positive for the presence of avian schistosomes. For these 11 samples, the 28S rDNA sequencing assay was able to identify an avian schistosome to the genus level in 10 samples (91%). The most common avian schistosome genus, *Trichobilharzia*, and avian schistosome lineage B, a schistosome related to *Gigantobilharzia huronensis* (Fig. 4), were identified from Mormon Island Lake in 2013. Both of these schistosomes are known to use the physid identified at Mormon Island Lake as intermediate hosts. *Gigantobilharzia huronensis* is a widely distributed North American avian schistosome hosted by passerine birds like grackles and blackbirds, but currently, we do not know the identity of the bird host for avian schistosome lineage B. *Trichobilharzia physellae*, another common physid-transmitted avian schistosome, was putatively identified from Twin Lake in Wisconsin. Therefore, on the basis of the results obtained from the assay and subsequent phylogenetic analysis, one may be more likely to encounter avian schistosome lineage B (whose avian host is as yet unknown) in physid snails at Mormon Lake in May and June. At Twin Lake, one may be more likely to encounter a species of *Trichobilharzia* transmitted by physid snails and ducks, likely either *T. physellae* or *T. querquedulae.* The recovery from Twin Lake of an opistorchiid sequence unrelated to schistosomes (Wisc6026) is not particularly surprising, as multiple nonschistosome digenetic trematode species are transmitted in such habitats, and their cercariae are often abundant in the water column. Consequently, some amplification of sequences from nontarget cercariae might be expected in complex environmental samples. Such caveats are not unexpected when assays involving sampling of large volumes of water potentially containing many diverse organisms are developed. Here we note that the follow-up 28S rDNA sequencing provides a straightforward way to correct for false-positive signals that may occur using the 18S rDNA TaqMan screening technique.

Mormon Island Lake and Twin Lake have repeatedly been associated with swimmer's itch over the years prior to this study. In 2012, suspected swimmer's itch cases associated with swimming in Mormon Island Lake were reported on 7 June. The lake was subsequently closed, but water samples for this study were not collected until 2 weeks later. During this 2-week period, snail populations on the recreational beach were observed to be dramatically reduced and bluegill fish were observed in significant numbers in the swimming area. The most common species of snail found was a *Physa* sp. Bluegill are known to eat snails, and large numbers of dead snails were observed in the beach area and in nearby bluegill beds. The relatively early date of the dermatitis outbreaks reported, followed by the appearance of many dead snails, may also suggest that the outbreaks were due to infected snails that had overwintered but, as is typical of many freshwater snails, died along with a cohort of the previous year's snails by early summer. The substantially reduced population of older snails in the lake may have been associated with the negative avian

TABLE 5 Avian schistosome detection by 18S rDNA TaqMan assay in lake water samples collected in Nebraska[a]

Sample identification no.	18S rDNA TaqMan assay result	Identification based on 28S rDNA sequencing
Neb4749	Neg.	None
Neb4750	Pos.	*Trichobilharzia* sp.
Neb4751	Pos.	*Trichobilharzia* sp.

[a] Samples were collected in 2014. Neg., negative by the 18S rDNA TaqMan assay; Pos., positive by the 18S rDNA TaqMan assay; None, no sequencing information was obtained. Only samples that were positive by the TaqMan assay were subjected to the 28S rDNA real-time PCR assay with SYTO 9 and sequenced. See Fig. 4 for phylogenetic identification.

schistosome water testing results obtained shortly after the initial early outbreak. Testing undertaken later in the summer (July to September), at a time sufficient to allow new infections to be acquired by and to mature in a new generation of snails, may have again yielded positive results. During the 2013 monitoring study of Mormon Island Lake, positive avian schistosome detection was obtained for a water sample collected on 29 May. Snails were not observed until 19 June, coinciding with increased and stronger positive detections in the water samples. The beach was closed for swimming between 5 June and 23 June 2013 to prevent swimmer's itch; no reported cases of swimmer's itch were associated with Mormon Island Lake in 2013. In 2014, initial reports of swimmer's itch were associated with a lake exposure that occurred on 28 May, and reports lasted through 15 June, when the beach was closed (Neb4750 and Neb4751; Fig. 4). Unlike the earlier samples following outbreaks, these species grouped with *Trichobilharzia* (Fig. 4). Further studies incorporating environmental testing and epidemiological investigations, especially covering the duration of the transmission season from spring through late summer, would increase the potential for correlating the findings of avian schistosome molecular testing and other environmental data with health risks to prevent and control swimmer's itch outbreaks.

This study demonstrates that a rapid TaqMan assay can effectively screen concentrated water samples for the presence of a diverse array of schistosomes and that subsequent 28S rDNA sequence analysis can identify schistosomes in the samples. For future deployment of this technique, it will be important to monitor the ability of the 28S rDNA assay to obtain good-quality sequences from environmental samples, as there is a possibility that chimeric sequences can occur in the presence of cercariae of multiple species or even genera within the samples. Depending on the goals of the screening program to be initiated, we also note that investigators in other areas or with different goals could easily substitute different primers to amplify sequences other than the 28S rDNA sequences that we targeted in the follow-up to the initial 18S rDNA TaqMan screen. In fact, as the schistosome sequence database becomes more complete, further evolution of this general approach is to be expected. As avian schistosomes often have defined patterns of host use, even provisional identification of the specific schistosomes involved in outbreaks provides important clues regarding the specific bird or snail species involved in contaminating the environments in the first place. The method developed here may also be useful to detect and identify avian schistosomes in lesions suffered by individuals with dermatitis if confirmation of swimmer's itch or ruling out of other causes of dermatitis was necessary. Swimmer's itch is by no means infrequent, and for many people, avian schistosomes may represent one of the few exposures to helminth antigens that they will receive in their lifetime in North America. Better tools to detect the cercariae involved will likely show that the potential for dermatitis is even more common than generally recognized, including across much of the developed world. Development of water testing and molecular tools will assist state public health and natural resources agencies in their response to suspected outbreaks and enable them to improve prevention and response activities to reduce the public health and economic impacts of swimmer's itch on communities.

ACKNOWLEDGMENTS

We thank Yvonne Qvarnstrom for the DNA extracts of *Angiostrongylus cantonensis* parasitic nematodes and noninfected slug hosts. We thank Sarah Sewall for collecting water samples from Twin Lake in Wisconsin. We thank Ramesh Devkota for the provision of samples of nonhuman mammalian schistosomes from Nepal.

The findings and conclusions in this report are those of the authors and do not necessarily represent those of the CDC or of the National Institutes of Health. Use of trade names and commercial sources is for identification only and does not imply endorsement by CDC or the U.S. Department of Health and Human Services.

This study was supported by a National Science Foundation grant to S.V.B. (DEB 1021427). Technical assistance at the University of New Mexico Molecular Biology Facility was supported by NIH grant P30GM110907 from the Institute Development Award program of the National Institute of General Medical Sciences. Specimens were provided by the Museum of Southwestern Biology, University of New Mexico.

REFERENCES

1. **Agrawal MC, Gupta S, George J.** 2000. Cercarial dermatitis in India. Bull World Health Organ **78:**278.
2. **Aldhoun JA, Kolarova L, Horak P, Skirnisson K.** 2009. Bird schistosome diversity in Iceland: molecular evidence. J Helminthol **83:**173–180. http://dx.doi.org/10.1017/S0022149X09289371.
3. **Brant SV, Loker ES.** 2009. Molecular systematics of the avian schistosome genus *Trichobilharzia* (Trematoda: Schistosomatidae) in North America. J Parasitol **95:**941–963. http://dx.doi.org/10.1645/GE-1870.1.
4. **Brant SV, Loker ES.** 2009. Schistosomes in the southwest United States and their potential for causing cercarial dermatitis or 'swimmer's itch.' J Helminthol **83:**191–198. http://dx.doi.org/10.1017/S0022149X09308020.
5. **Kolarova L, Skirnisson K, Horak P.** 1999. Schistosome cercariae as the causative agent of swimmer's itch in Iceland. J Helminthol **73:**215–220. http://dx.doi.org/10.1017/S0022149X99000335.
6. **Rao VG, Dash AP, Agrawal MC, Yadav RS, Anvikar AR, Vohra S, Bhondeley MK, Ukey MJ, Das SK, Minocha RK, Tiwari BK.** 2007. Cercarial dermatitis in central India: an emerging health problem among tribal communities. Ann Trop Med Parasitol **101:**409–413. http://dx.doi.org/10.1179/136485907X176463.
7. **Schets FM, Lodder WJ, van Duynhoven Y, Husman A.** 2008. Cercarial dermatitis in the Netherlands caused by *Trichobilharzia* spp. J Water Health **6:**187–195. http://dx.doi.org/10.2166/wh.2008.028.
8. **Soldanova M, Selbach C, Kalbe M, Kostadinova A, Sures B.** 2013. Swimmer's itch: etiology, impact, and risk factors in Europe. Trends Parasitol **29:**65–74. http://dx.doi.org/10.1016/j.pt.2012.12.002.
9. **Soleng A, Mehl R.** 2011. Geographical distribution of cercarial dermatitis in Norway. J Helminthol **85:**345–352. http://dx.doi.org/10.1017/S0022149X10000672.
10. **Valdovinos C, Balboa C.** 2008. Cercarial dermatitis and lake eutrophication in south-central Chile. Epidemiol Infect **136:**391–394. http://dx.doi.org/10.1017/S0950268807008734.
11. **Zbikowska E.** 2003. Is there a potential danger of "swimmer's itch" in Poland? Parasitol Res **89:**59–62. http://dx.doi.org/10.1007/s00436-002-0684-6.
12. **Baird JK, Wear DJ.** 1987. Cercarial dermatitis—the swimmers itch. Clin Dermatol **5:**88–91. http://dx.doi.org/10.1016/S0738-081X(87)80013-5.
13. **Kolarova L, Horak P, Skirnisson K, Mareckova H, Doenhoff M.** 2013. Cercarial dermatitis, a neglected allergic disease. Clin Rev Allergy Immunol **45:**63–74. http://dx.doi.org/10.1007/s12016-012-8334-y.
14. **Unckless RL, Makarewicz JC.** 2007. The impact of nutrient loading from Canada geese (*Branta canadensis*) on water quality, a mesocosm approach. Hydrobiologia **586:**393–401. http://dx.doi.org/10.1007/s10750-007-0712-8.
15. **Post DM, Taylor JP, Kitchell JF, Olson MH, Schindler DE, Herwig BR.** 1998. The role of migratory waterfowl as nutrient vectors in a managed wetland. Conserv Biol **12:**910–920. http://dx.doi.org/10.1046/j.1523-1739.1998.97112.x.
16. **Brant SV, Loker ES.** 2005. Can specialized pathogens colonize distantly related hosts? Schistosome evolution as a case study. PLoS Pathog **1:**167–169. http://dx.doi.org/10.1371/journal.ppat.0010038.
17. **Brant SV, Jouet D, Ferte H, Loker ES.** 2013. *Anserobilharzia* gen. n. (Digenea, Schistosomatidae) and redescription of *A. brantae* (Farr & Blankemeyer, 1956) comb. n. (syn *Trichobilharzia brantae*), a parasite of geese (Anseriformes). Zootaxa **3670:**193–206. http://dx.doi.org/10.11646/zootaxa.3670.2.5.
18. **Horak P, Mikes L, Lichtenbergova L, Skala V, Soldanova M, Brant SV.**

2015. Avian schistosomes and outbreaks of cercarial dermatitis. Clin Microbiol Rev **28:**165–190. http://dx.doi.org/10.1128/CMR.00043-14.
19. **Horak P, Kolarova L, Dvorak J.** 1998. *Trichobilharzia regenti* n. sp. (Schistosomatidae, Bilharziellinae), a new nasal schistosome from Europe. Parasite **5:**349–357. http://dx.doi.org/10.1051/parasite/1998054349.
20. **Haas W, Haeberlein S.** 2009. Penetration of cercariae into the living human skin: *Schistosoma mansoni* vs. *Trichobilharzia szidati*. Parasitol Res **105:**1061–1066. http://dx.doi.org/10.1007/s00436-009-1516-8.
21. **Haas W, van de Roemer A.** 1998. Invasion of the vertebrate skin by cercariae of *Trichobilharzia ocellata*: penetration processes and stimulating host signals. Parasitol Res **84:**787–795. http://dx.doi.org/10.1007/s004360050489.
22. **Horak P, Kolarova L, Adema CM.** 2002. Biology of the schistosome genus *Trichobilharzia*. Adv Parasitol **52:**155–233. http://dx.doi.org/10.1016/S0065-308X(02)52012-1.
23. **Brant SV, Loker ES.** 2013. Discovery-based studies of schistosome diversity stimulate new hypotheses about parasite biology. Trends Parasitol **29:**449–459. http://dx.doi.org/10.1016/j.pt.2013.06.004.
24. **Brant SV, Loker ES.** 2009. Etiology and epidemiology of cercarial dermatitis in North America, abstr 703. *In* Abstracts of the 58th Meeting of the American Society of Tropical Medicine and Hygiene. Am J Trop Med Hyg **81**(5 Suppl 1)**:**202. http://www.ajtmh.org/content/81/5_Suppl_1/201.full.pdf+html.
25. **Kane RA, Stothard JR, Rollinson D, Leclipteux T, Evraerts J, Standley CJ, Allan F, Betson M, Kaba R, Mertens P, Laurent T.** 2013. Detection and quantification of schistosome DNA in freshwater snails using either fluorescent probes in real-time PCR or oligochromatographic dipstick assays targeting the ribosomal intergenic spacer. Acta Trop **128:**241–249. http://dx.doi.org/10.1016/j.actatropica.2011.10.019.
26. **Hertel J, Hamburger J, Haberl B, Haas W.** 2002. Detection of bird schistosomes in lakes by PCR and filter-hybridization. Exp Parasitol **101:**57–63. http://dx.doi.org/10.1016/S0014-4894(02)00036-X.
27. **Schets FM, Lodder WJ, Husman AMD.** 2010. Confirmation of the presence of *Trichobilharzia* by examination of water samples and snails following reports of cases of cercarial dermatitis. Parasitology **137:**77–83. http://dx.doi.org/10.1017/S0031182009990849.
28. **Truett GE, Heeger P, Mynatt RL, Truett AA, Walker JA, Warman ML.** 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). Biotechniques **29:**52–54.
29. **Hill VR, Mull B, Jothikumar N, Ferdinand K, Vinje J.** 2010. Detection of GI and GII noroviruses in ground water using ultrafiltration and TaqMan real-time RT-PCR. Food Environ Virol **2:**218–224. http://dx.doi.org/10.1007/s12560-010-9049-y.
30. **Swofford DL.** 2002. PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4. Sinauer Associates, Sunderland, MA.
31. **Smith CM, Hill VR.** 2009. Dead-end hollow-fiber ultrafiltration for recovery of diverse microbes from water. Appl Environ Microbiol **75:**5284–5289. http://dx.doi.org/10.1128/AEM.00456-09.